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(57) Abstract

The present invention concerns a new assay which allows the identification of compounds which inhibit the formation of complexes between a product of the double minute 2 gene ("dm2") and p53 but not between p53 and DNA. Both the complex formation of labeled DNA, C-terminally truncated p53 and dm2 and disruption of dm2 from the labeled DNA-p53 complex by an inhibitor of an p53-dm2 interaction can be detected by a gel shift assay procedure. This assay permits the selection of compounds which, besides their inhibitory property, do not alter p53 specific DNA binding and do not disturb p53 conformation required for DNA binding or formation of active tetramer.

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ASSAY FOR IDENTIFYING INHIBITORS OF THE INTERACTION BETWEEN PROTEINS P53 AND DM2

The present invention relates to an assay for testing inhibitors of the interaction between proteins p53 and hdm2.

The protein encoded by the human double minute 2 gene, hdm2, forms a complex with the tumor suppressor gene product p53 both *in vitro* and *in vivo*. In some human cancers hdm2 is overexpressed and binds most of the cellular p53. Formation of this complex is favoring nucleoplasmic transformation since the complexed p53 looses the tumor suppressor activity. Compounds which prevent the interaction between p53 and hdm2 will release p53, promoting its tumor suppression activity within these cancerous cells. Similar results could also be obtained with animal cancer cells, e.g. in mouse. The mouse homologue to hdm2 is mdm2.

To search for inhibitors of p53 - hdm2 interaction, a high throughput primary binding assay, for example ELISA, can be used to select compounds and to initiate a medicinal chemistry program. However, assays which can be used for such a primary screening of p53-hdm2 binding have the disadvantage that artefacts may occur, i.e. wrong positive reaction can be obtained because of artefactual results due to the chemical properties of the tested substances.

In addition, compounds which inhibit the interaction between p53 and hdm2 can also alter p53 specific DNA binding, which is a totally undesired effect because DNA binding is a prerequisite of p53 tumor suppressor activity.

For example, p53 is sensible to several chemical agents that inhibit its activity. The main criteria of activity of p53 is the DNA binding which reveals that the protein is properly folded and not aggregated or unfolded. Compounds which like metal chelators precipitate p53 might be considered as true inhibitors of the p53 - hdm2 interaction in a classical binding assay because the precipitated p53 cannot form complexes.

Therefore, additional testing of the impact of the substance on p53-DNA binding is important because compounds inhibiting p53-DNA binding are not good candidates for therapeutic uses. However, in a high through put assay it is not possible to test

whether a compound which inhibits p53-hdm2 interaction prevents p53 specific DNA binding or disturbs the p53 conformation so that p53 can no more fulfill the desired biological function.

To avoid these two problems and to start a chemistry program based on more relevant lead compounds, the use of a good confirming assay is crucial.

A confirming assay according to the present invention could, for example, be a gel shift assay. A gel shift of a p53-DNA complex in an agarose gel after incubation with adeno-virus E1B protein is described in Yew et al. [Genes & Dev. 8, 190-202, 1994].

In Wang et al. [PNAS 91, 2230-2234, 1994] the detection of the binding of HB virus X protein to p53 by measuring the inhibition of p53-DNA binding is described. However, none of the prior art publications describes an assay in which the DNA binding property of p53 remains if p53 is complexed with a double minute 2 protein.

So far, all the in vitro assays described in the literature to study the interaction between p53 and hdm2 are immunoprecipitation assays for testing the binding of hdm2 to p53 [Leng et al., Oncogene 10:1275(1995)]. None of these assays simultaneously show that hdm2 binds to p53 and does not disturb its specific DNA binding.

In the present invention it was surprisingly found that p53-DNA binding is maintained after complex formation with hdm2 and that it is possible to measure in one and the same reliable assay the effect of a substance on both the p53-hdm2 and p53-DNA binding.

Object of the invention

It is the object of the invention to provide a reliable test method for compounds which inhibit the formation of complexes between hdm2 and p53 but which do not inhibit binding of DNA to p53 or disturb the p53 conformation so that p53 can no more fulfill the desired biological function.

Summary of the invention

The present invention concerns a new assay which allows the identification of compounds which inhibit the formation of complexes between a product of the double

minute 2 gene ("dm2"), for example human hdm2 or mouse mdm2, and p53 but not between p53 and DNA. Both the complex formation of labeled DNA, C-terminally truncated p53 and dm2 and disruption of dm2 from the labeled DNA-p53 complex by an inhibitor of the p53-dm2 interaction can be detected by a gel shift assay procedure. This assay permits the selection of compounds which, besides their inhibitory property, do not alter p53 specific DNA binding and do not disturb p53 conformation required for DNA binding or formation of active tetramer.

The invention further concerns a test kit for testing the effect of a substance on the binding of a dm2 protein to p53, comprising (a) a p53 or functional equivalent thereof having DNA-binding, oligomerisation and hdm2-binding properties, (b) a hdm2 or functional equivalent thereof having the p53 binding domain, and (c) a DNA sequence specifically binding to the p53 binding domain.

Detailed description of the invention

The present invention concerns a test method for a substance inhibiting the formation of a complex between p53 and a product of the double minute 2 gene ("dm2"), for example human (h)dm2 or mouse (m)dm2, while not inhibiting the formation of a complex between p53 and DNA. The method comprises measuring complex formation in a mixture of p53, dm2 and DNA binding to p53 in the presence and in the absence of a substance to be tested. In the presence of the desired property of the tested substance, a complex between p53 and DNA is formed ("double complex"), while in the absence of the desired property either a complex between p53, DNA and dm2 ("triple complex"; if no inhibiting activity is present) or no complex (if the tested compound inhibits both the dm2-p53 and p53-DNA complex formation or if the tested substance destroys the p53 conformation so that it is no more DNA binding) is formed. While any method being able to discriminate between the different conditions (triple complex, double complex, no complex) is suitable for performing the present assay, in a preferred embodiment of the invention the assay performed is a gel shift assay.

Thus, the test system essentially comprises a p53, a dm2, and DNA. While the use of the human proteins or active variants thereof is most preferred, the invention is not limited to the use of the human proteins. The corresponding proteins from other species can also be used, e.g. from mouse. However, it is preferred that both the p53 and dm2 protein used in the assay originate from the same species.

For performing the present invention, a p53 protein must be used which both is able to bind DNA and dm2.

p53 according to the present invention can be a recombinant form of p53 or purified from the original organism. It is, however, not necessary to use a full length p53 for performing the present invention. Accordingly, the p53 form used herein also means any useful variant or fragment of p53, preferentially of human p53. The features of such a useful variant or fragment are clear from the description hereinafter.

For DNA binding, p53 must be able to form tetrameric complexes. Consequently, for DNA binding both an active p53 DNA binding domain (e.g. residues 102 - 292 of p53) and a p53 functional oligomerisation domain (e.g. residues 325 - 356 of p53) must be present in the p53 form used in the present invention. For improving DNA binding properties of p53, the protein can be activated by interaction with a specific antibody (for example the monoclonal antibody Pab421 known in the art which binds to the amino acid stretch between amino acid 372 and 380 of the human p53), phosphorylation by kinases (casein kinase II phosphorylating Ser392 of human p53 or protein kinase C phosphorylating Ser 370 and Ser 375 of human p53) or, more preferably, truncation of its C-terminus (deletion of maximal 38 amino acids of the C-terminus of the natural p53 sequence). An example for the latter is p53D30, i.e. natural p53 lacking the C-terminal 30 amino acids, used in the Examples hereinafter.

In another embodiment of the invention, a p53-DNA complex for studying inhibitors of dm2-p53 interaction can also be obtained by the use of high affinity binding DNA elements (like the RGC and the BC sequences described in Kern et al., Science 252:1708, 1991, and Halazonetis et al., EMBO J 12:1021, 1993, respectively).

p53 can be directly purified from various sources (for example from bacteria, baculovirus or mammalian cells).

For being able to form complexes with hdm2, a human p53 suitable for use in the present invention contains residues 1 to 52 of the natural p53 sequence, more preferentially residues: 18 to 23, even more preferentially residues 19, 22 and 23.

The concentration of p53 which is preferentially used in the present invention depends on the amount of dm2 used in the assay. Normally, a five fold excess of dm2 protein is

used. A very clear signal in detection of radioactive label is obtained with about 50 to 100 ng of p53D30. However, it is also possible to use higher amounts if it is possible to tolerate in the test assay that some of the proteins precipitate.

A dm2 protein for use in the present invention can be recombinant or purified from the original organism.

A dm2 protein for use in the present invention can be either the full length form or a truncated form or any hybrid protein which contains the minimal p53 binding domain of the dm2. dm2 in context with the present invention means preferentially a dm2 from the same species as the p53 used in the assay is derived from. In particular, if human p53 is used, a human dm2 (hdm2) or analogue thereof containing the minimal p53 binding domain is used, and if mouse p53 is used, a mouse dm2 (mdm2) or analogue thereof containing the minimal p53 binding domain is used. For hdm2, the region from residue 1 to residue 102 of the natural sequence is identified so far as minimal p53 binding domain. For example, in an embodiment of the invention a fusion protein consisting of the N-terminal 188 amino acids of hdm2 (comprising the p53 binding domain) and the full length glutathione S-transferase from Schistosoma japonicum prepared in the Examples (named herein G-M fusion protein) can be used for the assay. The fusion protein is obtainable by expressing the DNA encoding the N-terminal 188 amino acids of hdm2 in the expression vector pGEX-2T (Pharmacia).

The DNA element of the test system can be any DNA fragment which specifically binds to p53, e.g. such containing a p53 binding element degenerated or not. It can be a synthetic oligonucleotide, a DNA fragment isolated from living organisms or a DNA element inserted in a plasmid.

The optimal p53 to hdm2 ratio may vary depending on the purity and specific binding activity of the used proteins and, thus, should be determined for each protein variant used.

In the case that a gel shift assay is performed, the DNA element should be such that a DNA band can be detected which shifts in the gel when the DNA is incubated with p53. For detection, the DNA element can be either radiolabeled or possibly labeled by a non radioactive method.

For obtaining a satisfactory detection signal, p53 should be satured with DNA. For example, the K_D of full length p53 activated by antibody Pab421 is about $5x10^{-10}$ M

For 50 ng p53D30 used in the Examples, 0.1 to 0.5 pmole of DNA should be sufficient.

For gel shift assay, the gel can be an agarose gel or, preferably, a native polyacrylamide gel, preferably such having 4 to 5 % acrylamide.

The buffer can be any buffer in which p53 is active for specific DNA binding since the complex p53 - DNA is less stable than the p53 - hdm2 complex. Preferred buffers are HEPES in a concentration of 20 to 50 mM or buffered Tris solution in a concentration of 10 to 50 mM.

In a preferred embodiment the pH of the buffer is 7.1 to 8.0. In a preferred embodiment of the invention a salt is present in the buffer. If a salt is used, it should preferentially be KCl at 50 to 100 mM or NaCl at 50 to 175 mM. Moreover, the buffer can optionally contain a substance selected from Glycerol (up to 20 %), DTT (up to 0.5 mM), $MgCl_2$ (e.g. about 6 mM), $ZnSO_4$ (e.g. about 0.1. mM), ZnOAc (e.g. about 0.1 mM), detergent NP40 (up to 0.1 %), Triton X-100 (up to 0.1 %), bovine serum albumin (up to 1 mg/ml), EDTA (up to 1 mM), and a competitor DNA, e.g. poly dI-dC, poly dA-dT or salmon sperm DNA, e.g. in a concentration of 25 to 100 µg/ml.

The invention further concerns a test kit for testing the effect of a substance on the binding of a dm2 protein to p53, said test kit essentially comprising (a) a p53 or functional equivalent thereof having DNA-binding, oligomerisation and hdm2-binding properties, (b) a hdm2 or functional equivalent thereof having the p53 binding domain, and (c) a DNA sequence specifically binding to the p53 binding domain. The preferred ingredients of the test kit are as above. The test kit optionally contains instructions for its use.

The following examples are illustrative, however, should not be construed to limit the present invention.

Examples

A) Material

Molecular biology reactants are purchased from Promega except the Pfu polymerase which is obtained from Stratagene, the pGEX-2T vector from Pharmacia Biotech and the pET-3a vector from Novagen. Immunologicals are purchased from Oncogene Science. Polydeoxyinosinic-deoxycytidylic acid is obtained from Sigma and [^{33}P]γ-adenosine triphosphate (ATP) from Amersham. The synthetic oligonucleotides are purchased in a purified and desalted form from Microsynth. All other chemicals are from Merck.

B) Molecular biology

The DNA region of the hdm2 gene encoding the first 188 amino acids of the protein is obtained by Polymerase Chain Reaction (PCR) amplification of the hdm2 gene. The oligonucleotides used for PCR are designed such that a BamHI restriction site is introduced at the 5' extremity of the gene and an EcoRI restriction site at its 3' end (see hdm2 primer I and II with SEQ ID Nos 8 and 9, respectively). The PCR fragments digested by BamHI and EcoRI are ligated with a BamHI / EcoRI cleaved pGEX-2T vector. The resulting vector comprises a fusion gene consisting of the full length sequence of glutathione-S-transferase of S. japonicum, a linker sequence, and the N-terminal 188 amino acids of hdm2, in the 5' to 3' order. The complete gene is sequenced on both strands and the recombinant plasmid is introduced into *E. coli* strain BL21 (Novagen).

Glutathione-S-transferase protein (for control experiments) was obtained from E. coli strain BL21 (Novagen) transformed with pGEX-2T plasmid.

The human wild type p53 gene is used as a template for PCR to obtain the gene fragment encoding for residues 1 to 362 of the 392 amino acids of natural p53 (p53D30). The oligonucleotides used for PCR are designed such that a NdeI restriction site is introduced at the 5' end and a BamHI site at the 3' end (see p53D30 primer I and II with SEQ ID Nos 10 and 11, respectively).

The PCR fragments digested by NdeI and BamHI are ligated with a NdeI / BamHI cleaved pET-3a plasmid. The complete gene is sequenced and the expression plasmid is introduced into *E. coli* strain BL21(DE3)pLysS (Novagen).

C) Protein expression

For protein expression bacteria cultures are inoculated by a 100-fold diluted overnight culture and grown in Luria Broth medium in the presence of 100 µg ampicillin / ml at 37 °C to OD_{600} = 0.8. The cultures are then cooled on ice to room temperature, induced with 1 mM isopropyl-D-thiogalactopyranoside and grown for four additional hours at 27 °C. The cells are then harvested by centrifugation and the pellets flash frozen in liquid nitrogen and stored at -70 °C.

D) Purification of the hdm2 fusion protein and of the glutathione S-transferase protein

The cell pellets containing the glutathione S-transferase fusion protein of hdm2 (named "G-M" in the following) are resuspended in ice cold buffer A (0.5 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonylfluoride (PMSF) - pH = 7.3) and lysed with a French press at 1000 psi. After centrifugation, the soluble fraction is loaded onto a Glutathione Sepharose 4B column (Pharmacia Biotech) preequilibrated at 4 °C with buffer A. The G-M fusion protein is then eluted with buffer B (50 mM Tris(hydroxymethyl)-aminomethane (Tris-HCl), 10 mM reduced glutathione, 0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM 2-mercaptoethanol - pH = 8.0). The fractions containing the protein are identified by SDS-PAGE (sodium dodecyl sulfate polyacryalmide gel electrophoresis), pooled and desalted on a Sephadex G25 column (Pharmacia Biotech) which is preequilibrated at 4 °C with buffer C (50 mM Tris.HCl, 50 mM NaCl, 20 % (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.1 % (v/v) Triton X-100 - pH = 7.6). The protein solution is loaded onto a Mono Q column (Pharmacia Biotech), preequilibrated with buffer C at 4 °C, and the fusion protein eluted with a linear gradient of buffer C containing 1 M NaCl. The fractions containing the purified G-M protein are pooled, concentrated (Centricon 30 - Amicon) to 1 mg / ml, flash frozen in liquid nitrogen and stored at -70 °C.

The glutathione S-transferase protein (named "G" in the following) is purified in the same procedure except that the purification is stopped after the Glutathione Sepharose 4B column due to the high purity of the material obtained after this step.

E) Purification of p53D30 protein

The cell pellets containing the p53D30 protein are resuspended in ice cold buffer D (50 mM 4-(2-hydroxyethyl)-piperazine-ethane-sulfonic acid (Hepes.NaOH), 10 % (v/v) glycerol, 0.1 mM EDTA, 0.1 % (v/v) Triton X-100, 5 mM 1,4-dithio-DL-threitol (DTT), 1 mM PMSF - pH = 7.6) and lysed with a French press at 1000 psi. After centrifugation, the soluble fraction is loaded onto a HiTrap Heparin column (Pharmacia Biotech) preequilibrated at 4 °C with buffer D. The column is first washed with buffer D containing 22 % buffer E (50 mM Hepes.NaOH, 1 M KCl, 10 % (v/v) glycerol - pH = 7.6) and p53D30 is eluted with a linear gradient to 100 % buffer E. The fractions containing p53D30 are pooled and loaded onto a HiTrap metal chelation column (Pharmacia Biotech) charged with nickel and preequilibrated at 4 °C with buffer F (50 mM Hepes.NaOH, 0.5 M KCl, 10 % (v/v) glycerol - pH = 7.6). After washing the column with buffer F containing 20 % buffer G (50 mM Hepes.NaOH, 0.5 M KCl, 10 % (v/v) glycerol, 0.1 M immidazole - pH = 7.6), p53D30 is eluted with 45 % buffer G. 50 mM 2-mercaptoethanol and 1 mM $ZnCl_2$ are added to the solution and the protein is dialysed at 4 °C against 50 mM Hepes.NaOH, 0.5 M KCl, 20 % (v/v) glycerol, 50 mM 2-mercaptoethanol, 1 mM $ZnCl_2$ - pH = 7.6. p53D30 is concentrated to 1 mg / ml (Amicon 30 kDa cut off membrane), flash frozen in liquid nitrogen and stored at - 70 °C.

F) Protein analysis

The purity of the protein preparation is evaluated by gel scanning (Schimadzu CS-930) on a SDS-PAGE (Laemmli, U.K. (1970) Nature, 227, 680-385) stained with Coomassie blue. Protein concentration is determined according to Bradford, M.B. (1976) Anal. Biochem., 72, 248-254).

G) Peptide synthesis

Peptide A (Ac-SQETFSDLWKL) shown in SEQ ID No. 5 is assembled on a Milligen 9050 automated peptide synthesizer (continuos flow) by solid - phase peptide synthesis using the fluorenylmethoxycarbonyl (Fmoc) strategy on Fmoc - MBHA - PAL - PEG amid resin. Side - chain protection of a-Fmoc amino acids is as follows: Asp(O-*tertio*butyloxycarbonyl), Gln(Trt), Glu(O-tertiobutyloxycarbonyl), Lys (butyloxycarbonyl), Ser(*tertio*butyl), Trp(butyloxycarbonyl), Thr(*tertio*butyl). The a-Fmoc amino acids (3

equivalents) are incorporated using the respective trichlorophenyl esters. Each coupling step is followed by an end - capping step (Ac_2O / pyridine in dimethyl formamide). After completion of the chain assembly, the dried peptide resin is treated with 76 % (v/v) trifluroacetic acid (TFA) / 20 % (v/v) EDT / 4 % (v/v) water at 30 °C in order to cleave the peptide from the resin and to deblock the side - chain protection. After 3 h incubation, the resin is separated by filtration and the peptide precipitated in cold (0 °C) *tert*-butyl-methyl ether. The crude peptide is collected by centrifugation and purified by preparative reversed - phase medium - pressure liquid chromatography using a Vydac C_{18} column (acetonitrile - water gradient containing 0.1 % (v/v) TFA) to yield the final product. The purity and the correct mass of the peptide is verified by analytical reversed - phase high pressure liquid chromatography, FABMS and matrix - assisted laser desorption ionisation time - of - flight mass spectrometry.

To the determine Peptide A concentration in solution, the peptide is dissolved in 50 mM Tris.HCl - pH = 7.6 and incubated for 10 min at 37 °C. The solution is extensively mixed and incubated for 15 min on ice. The insoluble fraction is eliminated by centrifugation at 13000 rpm for 10 min and the peptide concentration is determined by spectrophotometry at 280 nm using a molecular extinction coefficient of 5690.

H) Gel shift assay

Oligonucleotides I and II (SEQ ID Nos. 6 and 7, respectively) containing the 20-mer p53 consensus DNA binding site and HindIII-compatible ends are hybridised and 5' end-labelled with [^{33}P]γ- ATP as described in Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York.

A 20 µl reaction volume containing 50 mM Tris.HCl, 50 mM NaCl, 5 % (v/v) glycerol, 0.1 % (v/v) Triton X-100, 10 mM DTT and 50 µg / ml polydeoxyinosinic-deoxycytidylic acid - pH = 7.6 (binding buffer) are incubated for 30 min at 22 °C in the presence of the indicated amounts of p53D30, of radiolabelled oligonucleotides and of the mentioned monoclonal antibodies. Reactions are loaded onto a native 4 % polyacrylamide gel containing 0.5 x Tris.HCl - boric acid - pH = 8.0 which had undergone pre-electrophoresis at 200 V for 45 min at 4 °C. Electrophoresis is

continued at 200 V from 90 to 120 min at 4 °C. Gels are dried prior exposure to X-ray film (Amersham Hyperfilm-MP).

To perform the p53D30 - G-M - DNA ternary complex both proteins and the radiolabeled DNA are incubated in binding buffer for 30 min at 22 °C and the gel is proceeded as described previously.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CIBA-GEIGY AG
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(I) TELEX: 962 991

(ii) TITLE OF INVENTION: Assay

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1098 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:4..1089

(D) OTHER INFORMATION:/product= "Residue 1 to 362 of human p53 protein (named p53D30)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CAT ATG GAG GAG CCG CAG TCA GAT CCT AGC GTC GAG CCC CCT CTG AGT          48
    Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser
      1               5                  10                  15

CAG GAA ACA TTT TCA GAC CTA TGG AAA CTA CTT CCT GAA AAC AAC GTT          96
Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val
                20                  25                  30

CTG TCC CCC TTG CCG TCC CAA GCA ATG GAT GAT TTG ATG CTG TCC CCG         144
Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro
            35                  40                  45

GAC GAT ATT GAA CAA TGG TTC ACT GAA GAC CCA GGT CCA GAT GAA GCT         192
Asp Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala
        50                  55                  60

CCC AGA ATG CCA GAG GCT GCT CCC CCC GTG GCC CCT GCA CCA GCA GCT         240
Pro Arg Met Pro Glu Ala Ala Pro Pro Val Ala Pro Ala Pro Ala Ala
    65                  70                  75

CCT ACA CCG GCG GCC CCT GCA CCA GCC CCC TCC TGG CCC CTG TCA TCT         288
Pro Thr Pro Ala Ala Pro Ala Pro Ala Pro Ser Trp Pro Leu Ser Ser
80                  85                  90                  95

TCT GTC CCT TCC CAG AAA ACC TAC CAG GGC AGC TAC GGT TTC CGT CTG         336
Ser Val Pro Ser Gln Lys Thr Tyr Gln Gly Ser Tyr Gly Phe Arg Leu
                100                 105                 110
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GGC TTC TTG CAT TCT GGG ACA GCC AAG TCT GTG ACT TGC ACG TAC TCC      384
Gly Phe Leu His Ser Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser
            115                 120                 125

CCT GCC CTC AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC CCT GTG      432
Pro Ala Leu Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro Val
        130                 135                 140

CAG CTG TGG GTT GAT TCC ACA CCC CCG CCC GGC ACC CGC GTC CGC GCC      480
Gln Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val Arg Ala
    145                 150                 155

ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG AGG CGC      528
Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg
160                 165                 170                 175

TGC CCC CAC CAT GAG CGC TGC TCA GAT AGC GAT GGT CTG GCC CCT CCT      576
Cys Pro His His Glu Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro
                180                 185                 190

CAG CAT CTT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG TAT TTG GAT      624
Gln His Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp
            195                 200                 205

GAC AGA AAC ACT TTT CGA CAT AGT GTG GTG GTG CCC TAT GAG CCG CCT      672
Asp Arg Asn Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro
        210                 215                 220

GAG GTT GGC TCT GAC TGT ACC ACC ATC CAC TAC AAC TAC ATG TGT AAC      720
Glu Val Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn
    225                 230                 235

AGT TCC TGC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC      768
Ser Ser Cys Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile
240                 245                 250                 255

ACA CTG GAA GAC TCC AGT GGT AAT CTA CTG GGA CGG AAC AGC TTT GAG      816
```

```
Thr Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu
                260                 265                 270

GTG CGT GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA GAG GAA GAG      864
Val Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu
            275                 280                 285

AAT CTC CGC AAG AAA GGG GAG CCT CAC CAC GAG CTG CCC CCA GGG AGC      912
Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser
        290                 295                 300

ACT AAG CGA GCA CTG CCC AAC AAC ACC AGC TCC TCT CCC CAG CCA AAG      960
Thr Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys
    305                 310                 315

AAG AAA CCA CTG GAT GGA GAA TAT TTC ACC CTT CAG ATC CGT GGG CGT     1008
Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg
320                 325                 330                 335

GAG CGC TTC GAG ATG TTC CGA GAG CTG AAT GAG GCC TTG GAA CTC AAG     1056
Glu Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys
                340                 345                 350

GAT GCC CAG GCT GGG AAG GAG CCA GGG GGG AGC TGAGGATCC              1098
Asp Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser
            355                 360
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 362 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
 1               5                  10                  15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
            20                  25                  30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp
        35                  40                  45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
    50                  55                  60

Arg Met Pro Glu Ala Ala Pro Pro Val Ala Pro Ala Pro Ala Ala Pro
65                  70                  75                  80

Thr Pro Ala Ala Pro Ala Pro Ala Pro Ser Trp Pro Leu Ser Ser Ser
                85                  90                  95

Val Pro Ser Gln Lys Thr Tyr Gln Gly Ser Tyr Gly Phe Arg Leu Gly
            100                 105                 110

Phe Leu His Ser Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser Pro
        115                 120                 125

Ala Leu Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro Val Gln
    130                 135                 140

Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val Arg Ala Met
145                 150                 155                 160

Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg Cys
                165                 170                 175

Pro His His Glu Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro Gln
            180                 185                 190
```

His Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp Asp
195 200 205

Arg Asn Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro Glu
210 215 220

Val Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser
225 230 235 240

Ser Cys Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr
245 250 255

Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu Val
260 265 270

Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn
275 280 285

Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr
290 295 300

Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys
305 310 315 320

Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg Glu
325 330 335

Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys Asp
340 345 350

Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser
355 360

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 729 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:84..647

(D) OTHER INFORMATION:/product= "N-terminal 188 amino acids of human double minute protein 2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
TAGCATGGCC TTTGCAGGGC TGGCAAGCCA CGTTTGGTGG TGGCGACCAT CCTCCAAAAT      60

CGGATCTGGT TCCGCGTGGA TCC ATG TGC AAT ACC AAC ATG TCT GTA CCT          110
                          Met Cys Asn Thr Asn Met Ser Val Pro
                                  365                 370

ACT GAT GGT GCT GTA ACC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG       158
Thr Asp Gly Ala Val Thr Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu
            375                 380                 385

ACC CTG GTT AGA CCA AAG CCA TTG CTT TTG AAG TTA TTA AAG TCT GTT       206
Thr Leu Val Arg Pro Lys Pro Leu Leu Leu Lys Leu Leu Lys Ser Val
        390                 395                 400

GGT GCA CAA AAA GAC ACT TAT ACT ATG AAA GAG GTT CTT TTT TAT CTT       254
Gly Ala Gln Lys Asp Thr Tyr Thr Met Lys Glu Val Leu Phe Tyr Leu
    405                 410                 415

GGC CAG TAT ATT ATG ACT AAA CGA TTA TAT GAT GAG AAG CAA CAA CAT       302
Gly Gln Tyr Ile Met Thr Lys Arg Leu Tyr Asp Glu Lys Gln Gln His
420                 425                 430                 435
```

ATT GTA TAT TGT TCA AAT GAT CTT CTA GGA GAT TTG TTT GGC GTG CCA 350
Ile Val Tyr Cys Ser Asn Asp Leu Leu Gly Asp Leu Phe Gly Val Pro
440 445 450

AGC TTC TCT GTG AAA GAG CAC AGG AAA ATA TAT ACC ATG ATC TAC AGG 398
Ser Phe Ser Val Lys Glu His Arg Lys Ile Tyr Thr Met Ile Tyr Arg
455 460 465

AAC TTG GTA GTA GTC AAT CAG CAG GAA TCA TCG GAC TCA GGT ACA TCT 446
Asn Leu Val Val Val Asn Gln Gln Glu Ser Ser Asp Ser Gly Thr Ser
470 475 480

GTG AGT GAG AAC AGG TGT CAC CTT GAA GGT GGG AGT GAT CAA AAG GAC 494
Val Ser Glu Asn Arg Cys His Leu Glu Gly Gly Ser Asp Gln Lys Asp
485 490 495

CTT GTA CAA GAG CTT CAG GAA GAG AAA CCT TCA TCT TCA CAT TTG GTT 542
Leu Val Gln Glu Leu Gln Glu Glu Lys Pro Ser Ser Ser His Leu Val
500 505 510 515

TCT AGA CCA TCT ACC TCA TCT AGA AGG AGA GCA ATT AGT GAG ACA GAA 590
Ser Arg Pro Ser Thr Ser Ser Arg Arg Arg Ala Ile Ser Glu Thr Glu
520 525 530

GAA AAT TCA GAT GAA TTA TCT GGT GAA CGA CAA AGA AAA CGC CAC AAA 638
Glu Asn Ser Asp Glu Leu Ser Gly Glu Arg Gln Arg Lys Arg His Lys
535 540 545

TCT GAT AGT TGAGAATTCA TCGTGACTGA CTGACGATCT GCCTCGCGCG 687
Ser Asp Ser
550

TTTCGGTGAT GACGGTGAAA ACCTCTGACA CATGCAGCTC CC 729

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 188 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
 1               5                  10                  15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
            20                  25                  30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
        35                  40                  45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
    50                  55                  60

Arg Leu Tyr Asp Glu Lys Gln Gln His Ile Val Tyr Cys Ser Asn Asp
65                  70                  75                  80

Leu Leu Gly Asp Leu Phe Gly Val Pro Ser Phe Ser Val Lys Glu His
                85                  90                  95

Arg Lys Ile Tyr Thr Met Ile Tyr Arg Asn Leu Val Val Val Asn Gln
            100                 105                 110

Gln Glu Ser Ser Asp Ser Gly Thr Ser Val Ser Glu Asn Arg Cys His
        115                 120                 125

Leu Glu Gly Gly Ser Asp Gln Lys Asp Leu Val Gln Glu Leu Gln Glu
    130                 135                 140

Glu Lys Pro Ser Ser Ser His Leu Val Ser Arg Pro Ser Thr Ser Ser
```

145 150 155 160

Arg Arg Arg Ala Ile Ser Glu Thr Glu Glu Asn Ser Asp Glu Leu Ser
165 170 175

Gly Glu Arg Gln Arg Lys Arg His Lys Ser Asp Ser
180 185

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "N-acetyl serine in position 1"
/label= modifiedsite

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: protein_bind

(B) LOCATION:5..26

(D) OTHER INFORMATION:/bound_moiety= "p53 consensus binding site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGCTTAGACA TGCCTAGACA TGCCTA 26

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: protein_bind

(B) LOCATION:5..26

(D) OTHER INFORMATION:/bound_moiety= "p53 consensus binding site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGCTTAGGCA TGTCTAGGCA TGTCTA 26

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..34
(D) OTHER INFORMATION:/product= "PCR primer for cloning hdm2 N-terminal 188 aminoacid coding"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GATCCGGGAT CCATGTGCAA TACCAACATG TCTG 34

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION:1..38

(D) OTHER INFORMATION:/product= "PCR primer for cloning hdm2 188 N-terminal amino acid coding"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCCGGAAT TCTCAACTAT CAGATTTGTG GCGTTTTC 38

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION:1..31

(D) OTHER INFORMATION:/product= "PCR primer for p53D30 cloning"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATCCGCATA TGGAGGAGCC GCAGTCAGAT C 31

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..36
(D) OTHER INFORMATION:/product= "PCR primer for p53D30 cloning"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCCGGGAT CCTCAGCTCC CCCCTGGCTC CTTCCC 36

Claims:

1. A method for testing the effect of a substance on the binding of a dm2 protein to p53, characterized in that complex formation is investigated in a mixture comprising (a) a p53 or functional equivalent thereof having specific DNA-binding, oligomerisation and dm2-binding properties, (b) a dm2 or functional equivalent thereof having the p53 binding domain, (c) a DNA sequence specifically binding to the p53 specific DNA binding domain, and (d) the substance to be tested.

2. The method according to claim 1 characterized in that complex formation is tested by gel shift assay.

3. The method according to claim 1 characterized in that human p53 is used.

4. The method according to claim 1 characterized in that the p53 used is p53D30.

5. The method according to claim 1 characterized in that hdm2 is used.

6. The method according to claim 1 characterized in that the functional equivalent is a truncated dm2 with p53 binding properties is used.

7. The method according to claim 1 characterized in that the functional equivalent is a protein which comprises the N-terminal 188 amino acids of hdm2 .

8. The method according to claim 1 characterized in that the functional equivalent is a fusion protein comprising glutathion-S-transferase of S. japonicum and the N-terminal 188 amino acids of hdm2.

9. The method of claim 1 in which both the binding of hdm2 to p53 and the binding of p53 to DNA is tested at the same time.

10. A test kit for testing the effect of a substance on the binding of a dm2 protein to p53, comprising (a) a p53 or functional equivalent thereof having DNA-binding, oligomerisation and hdm2-binding properties, (b) a hdm2 or functional equivalent thereof having the p53 binding domain, and (c) a DNA sequence specifically binding to the p53 binding domain.

INTERNATIONAL SEARCH REPORT

Inter ·ional Application No
PC1/EP 96/03957

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/53 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 20238 (UNIV JOHNS HOPKINS) 14 October 1993 ---	
A	EP,A,0 518 650 (UNIV JOHNS HOPKINS ;PHARMAGENICS INC (US)) 16 December 1992 ---	
A	GENES & DEVELOPMENT, vol. 8, no. 2, January 1994, pages 190-202, XP000564411 P. R. YEW ET AL.: "Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53." cited in the application --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 1996	21. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Cartagena y Abella,P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL. ACAD. SCI., vol. 91, 15 March 1994, USA, pages 2230-2234, XP000611682 X. WEI WANG ET AK.: "Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3." cited in the application ---	
A	ONCOGENE, vol. 10, no. 7, 6 April 1995, pages 1275-1282, XP000610901 P. LENG ET AL.: "N-terminal 130 amino acids ofMDM2 are sufficient to inhibit p53-mediated transcriptional activation." cited in the application -----	